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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Diabetes mellitus is a devastating metabolic disease characterized by hyperglycemia that can occur through distinct mechanisms, such as islet β-cell destruction, β-cell failure, and insulin resistance in peripheral tissues. The increasing prevalence of diabetes also affects the military personnel. Several genes associated with diabetes have been identified in genome wide association studies, but their genetic validation as causative factors remains largely unexplored. In this grant proposal, we focused on one of these genes, *Twist1*, whose conditional overexpression in the pancreatic tissue led to severe hyperglycemia and diabetes. We proposed experiments to investigate how Twist1 induces diabetes. The results showed that conditional overexpression of Twist1 in pancreatic progenitor cells in mice resulted in fatty pancreas development, leading to pancreatic insufficiency and diabetes. Mechanistic experiments demonstrate that Twist1 drives fatty pancreas formation by selectively reprogramming acinar cells towards the adipocyte lineage. Besides driving fatty pancreas formation, Twist1 also suppresses expression of Pdx1, the master transcription factor in β-cell, which drives expression of insulin. Loss-of-function genetic experiments demonstrated that *Twist1* deletion against fatty pancreas formation driven by obesity, thereby improving glucose tolerance and diabetes. Overall, these findings implicate Twist1 as a possible target for attenuating diabetes associated with obesity.

15. SUBJECT TERMS
None provided.

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1. INTRODUCTION

Diabetes is a complex disease caused by abnormal expression of multiple genes that govern critical aspects of pancreatic development and homeostasis. Several potential genes associated with diabetes have been identified by integrative genomic approaches, yet their contribution to the pathogenesis of diabetes remains to be established. In this proposal, we intended to focus our efforts on one of these candidate diabetes genes, *Twist1*, which encodes for a transcription factor known to govern fundamental biological processes crucial for proper body development and maintenance of organ homeostasis throughout life. Our preliminary data showed that enforced expression of Twist1 in the pancreatic epithelium in mice resulted in abnormal pancreatic development and hyperglycemia, reminiscent of diabetes. Based on this novel observation, we hypothesized that Twist1 overexpression might affect insulin production by islets β-cells, thereby culminating in insulin insufficiency and attendant hyperglycemia and diabetes. To test this overreaching hypothesis, we proposed to conduct genetic experiments using mice harboring either conditional overexpression (*Twist1*+) or conditional knockout of *Twist1* (*Twist1.KO*) in the pancreatic tissue. We designed several approaches to conduct full analyses of the diabetic phenotype of *Twist1*+ mice, including pancreas histology, blood glucose level, serum insulin levels, glucose tolerance, insulin tolerance and β-cell mass, proliferation, apoptosis and dedifferentiation. To corroborate the role of endogenous *Twist1* in driving β-cell dysfunction, we proposed to challenge *Twist1.KO* mice with high fat diet and carry out the same experiments as described earlier for *Twist1*+ mice. To delineate the molecular mechanisms by which Twist1 affects β-cell homeostasis and insulin production, we designed molecular and biochemical studies aimed at elucidating whether Twist1 functions to repress transcription of the *Pdx1* gene (pancreatic duodenal homeobox-1), which encodes for a master transcription factor that directly regulate synthesis and production of insulin by islet β-cells. To the best of our knowledge, this was the first study to show that Twist1 plays a crucial role in β-cell function and glucose homeostasis.

2. KEYWORDS

- Diabetes mellitus
- Hyperglycemia
- Obesity
- Genome wide association studies
- Transcription factor Twist1
- Pancreas-specific overexpression of *Twist1*
- Pancreas-specific deletion of *Twist1*
- Pancreatic progenitor cells
- Transcription factor Pdx1 (pancreatic duodenal homeobox-1)
- Insulin production
- Islet β-cells
- Fatty pancreas
- Acinar compartment
- Ductal compartment

3. ACCOMPLISHMENTS

3.A. What were the major goals of the project?

The observation that motivated our study on the role of Twist1 in diabetes was serendipitous. We found that overexpressing Twist1 in pancreatic progenitor cells in mice resulted in abnormally small pancreas, which is accompanied by severe hyperglycemia and diabetes. Extending our analysis to human diabetes using public SNP-based GWAS data, we found that *TWIST1* was significantly associated with diabetes. Moreover, gene microarray analysis showed that *TWIST1* is more expressed in obese diabetic patients than in obese healthy people (p=0.035). In efforts to unravel the molecular mechanisms by which Twist1 affects pancreas homeostasis, we found that overexpressing Twist1 induced decreased expression of the Pdx1 protein, a key mediator of β-cell development and insulin production, whose deregulation has been shown to be associated with diabetes. The *Pdx1* promoter possesses two conserved Twist1 E-Box binding motifs, raising the possibility that Twist1 might function as a direct transcriptional repressor of the *Pdx1* gene. Since Twist1 represses expression of its target genes through recruiting HDACs to chromatin, we turned our attention on HDAC9, which is a key regulator of β-cell differentiation. SNP analysis using GWAS data clearly indicated that HDAC9 is associated with diabetes (p= 5.9E-6), a hint that was further supported by gene microarray datasets highlighting elevated expression of HDAC9 in diabetic patients as compared to healthy individuals (p=0.03). In light of these observations, we hypothesize that Twist1 may repress *Pdx1* expression, thereby compromising β-cell function, which in turn culminates in defective insulin production and attendant diabetes development. We proposed to test this hypothesis as described in our statement of work:

Major Task 1: Characterization of the diabetic phenotype of mice bearing conditional overexpression of *Twist1*

MT1-Subtask 1: Evaluation of hyperglycemia and serum insulin abundance in mice with conditional overexpression of *Twist1* in the pancreatic tissue (*Twist1*+). Blood glucose level will be determined using the Relizon system and serum insulin level will be determined using an ELISA kit.

MT1-Subtask 2: Glucose tolerance test using *Twist1*+ mice. Glucose (2g/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the Relizon system.

MT1-Subtask 3: Insulin tolerance test using *Twist1+* mice. Insulin (1U/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) using the Relizon system.

MT1-Subtask 4: Evaluation of β-cell mass, proliferation, apoptosis and dedifferentiation in *Twist1+* mice. Pancreatic tissues will be fixed in formalin, embedded in paraffin and sections will be subjected to immunofluorescence using appropriate antibodies.

MT1-Milestone(s) Achieved: The study will provide compelling evidence that enforced expression of Twist1 affects β-cell development, leading to hyperglycemia and diabetes.

Major Task 2: Investigate the role of endogenous Twist1 in driving β-cell failure in *Twist1* conditional knockout mice undergoing high fat-induced diabetes.

MT2-Subtask 1: Generation of experimental mice harboring pancreas-specific *Twist1* knockout (*Twist1.KO*) by crossbreeding the breeding pairs *Twist1.fl/fl;Pdx1.Cre* mice and *Twist1.fl/fl* mice.

MT2-Subtask 2: Exposure of *Twist1.KO* mice to high fat diet and assessment of hyperglycemia and serum insulin abundance. Blood glucose level will be determined using the Relizon system and serum insulin level will be determined using an ELISA kit.

MT2-Subtask 3: Glucose tolerance test using *Twist1.KO* mice. Glucose (2 g/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the Relizon system.

MT2-Subtask 4: Insulin tolerance test using *Twist1.KO* mice. Insulin (1U/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the Relizon system.

MT2-Subtask 5: Evaluation of β-cell mass, proliferation, apoptosis and dedifferentiation in *Twist1.KO* mice subjected to high fat diet. Pancreatic will be fixed in formalin, embedded in paraffin and sections subjected to immunofluorescence using appropriate antibodies.

MT2-Milestone(s) Achieved: Completion of these experiments will provide strong evidence that endogenous Twist1 plays crucial roles in β-cell dysfunction under diabetic conditions resulting from exposure to high-fat diet.

Major Task 3: Investigate the molecular mechanisms by which Twist1 represses *Pdx1* expression

MT3-Subtask 1: Quantification of *Pdx1* mRNA and protein by qRT-PCR and Western blotting, respectively. We will use pancreatic tissues from *Twist1+* or *Twist1.KO* mice and their appropriate control. In addition, we will perform gene reporter assays using Min6 cells transfected with *Pdx1-Luc* in the absence or presence of Twist1 or siRNAs targeting Twist1.

MT3-Subtask 2: Evaluation of binding of Twist1 to the *Pdx1* promoter by chromatin immunoprecipitation (ChIP) and pull-down assays. We will use chromatin extracts or protein lysates from Min6 cells and confirm the results using chromatin extracts or protein lysates from *Twist1+* or *Twist1.KO* and their appropriate controls.

MT3-Subtask 3: Delineate the molecular mechanisms by which Twist1 represses expression of *Pdx1*. For this, we will investigate the interaction between *Twist1* and *HDAC9* using a combination of coimmunoprecipitation or ChIP approaches. We will use chromatin extracts or protein lysates from Min6 cells and confirm the results using chromatin extracts or protein lysates from *Twist1+* or *Twist1.KO* and their appropriate controls.

MT3-Milestone(s) Achieved: Achievement of this study will provide compelling evidence that Twist1 affects β-cell development through its ability to repress expression of *Pdx1*, which encodes the master transcription factor in β-cells. In addition, our findings will indicate whether Twist1 represses *Pdx1* expression by recruiting HDAC9, a general transcriptional repressor that plays an important role in the pathogenesis of diabetes

3.B. What was accomplished under these goals?

To test our overarching hypothesis, we proposed to develop the following specific aims:

Specific Aim 1: **Conduct comprehensive characterization of Twist1's ability to drive diabetes, with particular emphasis on blood glucose and serum insulin levels, glucose tolerance, and β-cell mass and function.**

Specific Aim 2: **Explore the mechanisms by which Twist1 affects β-cell development, focusing on its functional interaction with HDAC9 within the context of transcriptional repression of the *Pdx1* gene, which encodes the master transcription factor in β-cells.**

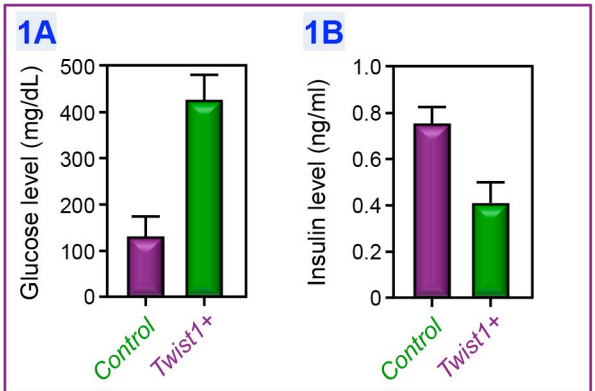
These specific aims remained unchanged during the entire funding period. Overall, we performed the vast majority of experiments described in our original grant application, and the data clearly showed

that Twist1 expression in the pancreatic tissue plays a major role in diabetes. Beyond this grant proposal, our new data also allowed us to progress significantly in another ongoing project related to the mechanisms by which Twist1 promotes pancreatitis and pancreatic cancer. In fact, we found that fatty pancreas formation induced by Twist1 also plays a major role in these two life-threatening conditions.

Results and Key findings

Pancreas-specific overexpression of Twist1 promotes fatty pancreas formation and diabetes

To investigate the role of Twist1 in pancreas development, we conducted genetic studies using mice with pancreas-specific overexpression of *Twist1* (*Twist1*+). Accordingly, we crossed mice bearing a Cre-activable *Twist1* transgene, *LCL-Twist1,* with *Pdx1.Cre* mice, which express Cre recombinase in all pancreatic progenitor cells. *Twist1*+ mice were born at the expected Mendelian ratio, showed no evidence of any gross anatomic or physiological abnormalities, and had normal weight at birth, indicating that Twist1 overexpression in the pancreas glandular did no affect early development. Intriguingly, measuring blood glucose of 4-week-old *Twist1*+ animals revealed severe hyperglycemia, which was associated with low circulating insulin levels (Figures 1A and 1B). At necropsy, *Twist1*+ mice displayed very undeveloped pancreas (Figure 2); the ratio of pancreas to whole-body weight did not exceed 20% of those of the wild-type littermates, suggesting that Twist1 overexpression might affect pancreatic development during postnatal life. In efforts to investigate the mechanisms behind this severe diabetic phenotype, we performed several experiments to analyze pancreas histology using *Twist1*+ and control mice of 4 to 8 weeks of age. As assessed by hematoxylin and eosin (H&E) staining, we detected a dramatic disorganization of the acinar parenchyma, which became entirely composed of cells with adipocyte morphology (Figure 3). Intriguingly, we



- **Fig.1A:** Blood glucose levels in control and *Twist1*+ mice (n=24).
- **Fig.1B:** Blood insulin levels in control and *Twist1*+ mice (n=6).

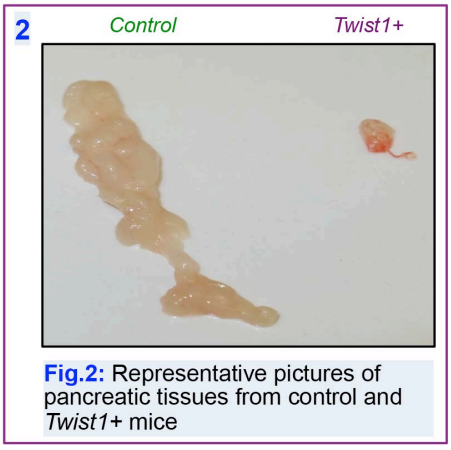


Fig.2: Representative pictures of pancreatic tissues from control and *Twist1*+ mice

observed the existence of structures resembling normal islets within this fatty pancreatic tissue, suggesting that Twist1 overexpression might affect specifically the acinar lineage (Figure 3). To confirm these findings, we conducted immunohistochemistry using antibodies to insulin, cytokeratin 19 (CK19, marker of ductal cells), and CPA1 (marker of acinar cells). We detected the presence of islets producing insulin within fatty pancreas (Figure 4), indicating that Twist1 overexpression did not affect directly the integrity of islet cells or differentiation of β-cells. However, the islets in Twist1^{+} were smaller in size when compared to those of the wild-type littermates (Figure 4), suggesting that Twist1 overexpression might affect β-cell proliferation. Of note, we detected normal duct structures within fatty pancreas (Figure 4), indicating that Twist1 overexpression did not affect the pancreatic ductal cell lineage. Collectively, these findings strongly suggest that Twist1 overexpression drives diabetes by affecting islet β-cells proliferation. In addition, our data illustrate that Twist1 overexpression promotes reprograming the acinar cells to the adipocyte lineage, which likely creates an inflammatory environment that further impinges on β-cell function, thereby leading to insulin insufficiency and attendant diabetes.

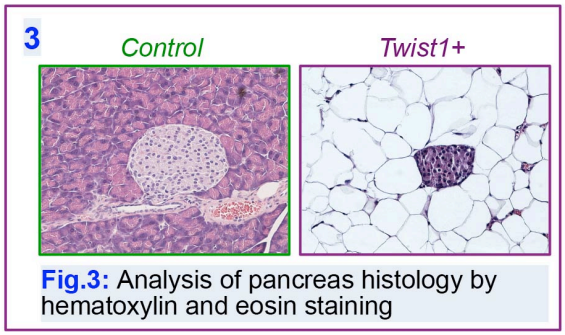


Fig.3: Analysis of pancreas histology by hematoxylin and eosin staining

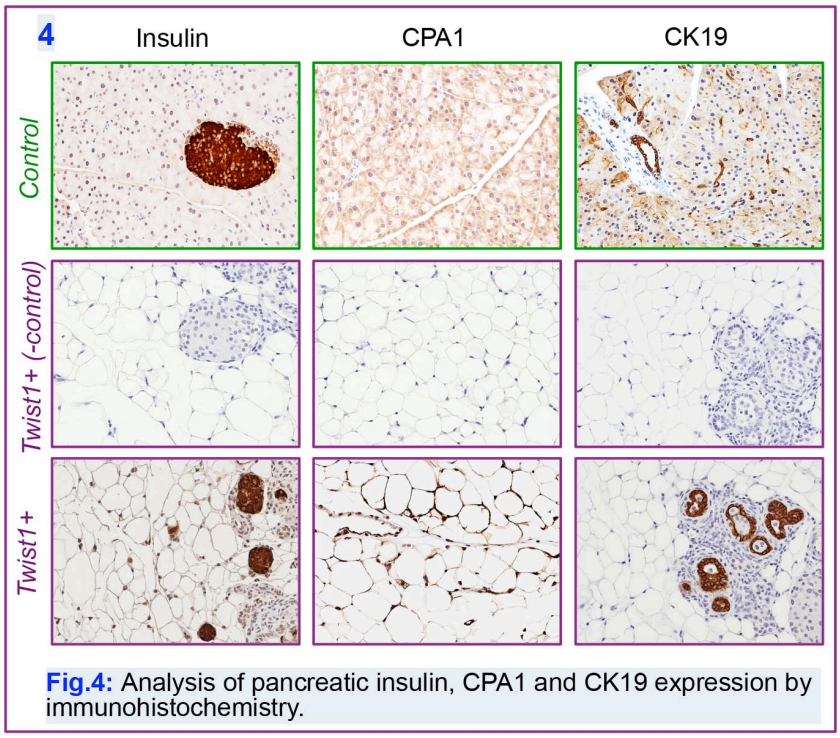


Fig.4: Analysis of pancreatic insulin, CPA1 and CK19 expression by immunohistochemistry.

Pancreas-specific deletion of Twist1 suppresses diabetes associated with fatty pancreas formation

To investigate the role of endogenous Twist1 in pancreatic function, we generated mice with pancreas-specific deletion of *Twist1* (*Twist1.KO*) as described earlier. As for *Twist1+* mice, *Twist1.KO* mice

were born at the expected Mendelian ratio, showed no evidence of any gross anatomic or physiological abnormalities, and had normal weight throughout postnatal life, indicating that Twist1 is dispensable for pancreas development. To investigate the role of endogenous Twist1 in diabetes, we challenged *Twist1.KO* mice with high-fat diet for 24 weeks to induce obesity and diabetes. Interestingly, although *Twist1.KO* mice gained similar weight as wild-type mice, they showed improved glucose clearance, as assessed by a glucose tolerance test (Figure 5). Histopathology analysis by hematoxylin and eosin staining showed that *Twist1* deletion almost completely blocked fatty pancreas formation driven by high-fat diet (Figure 6), providing further evidence that Twist1 mediates fatty pancreas formation, and further suggesting that fatty pancreas formation might play an important role in diabetes acquisition. As such, these experiments demonstrate that Twist1 plays an instrumental role in the pathogenesis of diabetes under obesity circumstances.

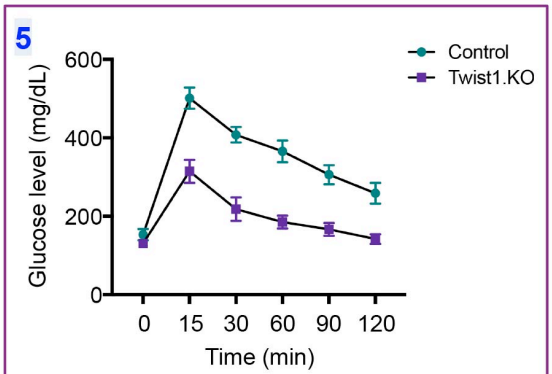


Fig.5: Glucose tolerance tests using control and *Twist1.KO* mice receiving high-fat diet for 24 weeks (n=6).

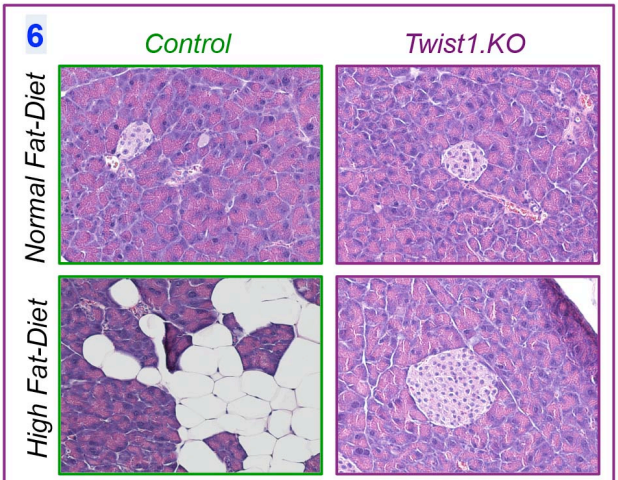
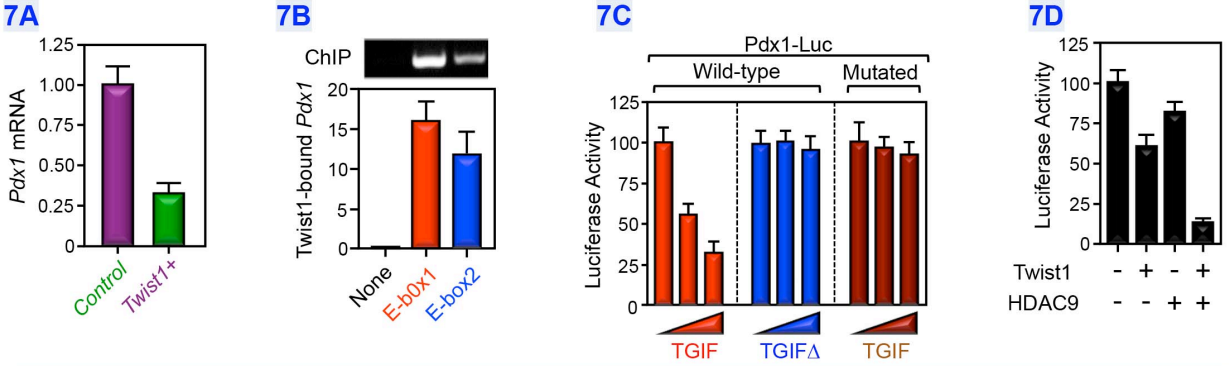


Fig.6: Analysis of pancreas histology by hematoxylin and eosin staining.

Twist1 functions as a direct transcriptional repressor for Pdx1

Our preliminary data showed that pancreas-specific overexpression of Twist1 led to a dramatic decrease in the expression of the Pdx1 protein, raising the intriguing possibility that Twist1 might inhibit insulin synthesis and secretion by islet β-cells. In this study, we undertook a variety of experimental approach to investigate whether Twist1 could directly repress expression of the *Pdx1* gene, and, if so, whether HDAC9 is involved in this process. First, we performed qRT-PCR using pancreatic tissues from *Twist1*+ and control mice, and detected a marked decrease in the expression of *Pdx1* mRNA in *Twist1*+ mice relative to control mice (Figure 7A). Second, an in silico search identified two consensus Twist1 binding sites (E-boxes) within the *Pdx1* promoter. To directly

investigate whether Twist1 could bind directly to the *Pdx1* promoter, we performed ChIP assays using pancreatic chromatin from *Twist1*+ and control mice. As shown in Figure 7B, we detected a dramatic increase in the binding of Twist1 to both E-box sites in the *Pdx1* promoter in *Twist1*+ mice when compared to control mice. Third, we assessed the ability of Twist1 to activate a luciferase reporter (Pdx1-Luc) driven by wild-type or mutated (E-box) variants of the *Pdx1* promoter. Using Min6 cells, we found that ectopic expression of Twist1 repressed luciferase expression exclusively from the wild-type Pdx1-Luc reporter (Figure 7C), confirming the presence of Twist1 responsive elements within the *Pdx1* promoter. Finally, gene reporter assays demonstrated that HDAC9 synergizes with Twist1 to inhibit Pdx1 expression (Figure 7D). In addition to these key findings, we obtained large amounts of in vitro and in vivo data showing that Twist1 functions in partnership with HDAC9 to repress expression of the *Pdx1* gene. Collectively, these findings provide strong evidence that Twist1 functions as a direct transcription repressor for *Pdx1*, thereby supporting a model in which Twist1 functions in islet β-cells to compromise insulin synthesis.



- **Fig.7A:** Analysis of *Pdx1* mRNA expression in control and *Twist1*+ mice by qRT-PCR.
- **Fig.7B:** Binding of Twist1 to the *Pdx1* promoter was analyzed by PCR or quantitative PCR using chromatin from control and *Twist1*+ mice.
- **Fig.7C:** Min6 cells were transfected with the indicated plasmids and examined by the Dual-Luciferase Reporter Assay System 48 hr after transfection.
- **Fig.7D:** Min6 cells were transfected with the indicated plasmids and examined by the Dual-Luciferase Reporter Assay System 48 hr after transfection.

3.C. What opportunities for training and professional development has the project provided?

A PhD student (Thien Ly Nguyen) in my lab is currently working on the role of Twist1 in diabetes and its link to other pancreatic diseases.

3.D. How were the results disseminated to communities of interest?

“Nothing to Report”

3.E. What do you plan to do during the next reporting period to accomplish the goals?

“Not Applicable”

4. IMPACT

4.A. What was the impact on the development of the principal discipline(s) of the project?

"Nothing to Report"

4.B. What was the impact on other disciplines?

Given that fatty pancreas is associated with other human diseases, such as chronic pancreatitis and pancreas cancer, we explored whether Twist1 could contribute to these phenotypes, which could help achieve a major research project of the lab dedicated to unravel mechanistic paradigms of these two devastating diseases. For instance, we found that *Twist1* deletion completely blocked fatty pancreas formation under both conditions, and this was mirrored by a striking improvement in diabetes of mice with chronic pancreatitis and survival of mice with pancreatic cancer. Based on these findings, we concluded that Twist1 is essential for fatty pancreas development irrespective of the disease conditions that drive that fatty pancreas phenotype. More importantly, these data shed tantalizing insights how Twist1 contributes to pancreatitis and pancreatic cancer in addition to diabetes.

4.C. What was the impact on technology transfer?

"Nothing to Report"

4.D. What was the impact on society beyond science and technology?

"Nothing to Report"

5. CHANGES / PROBLEMS

5.A. Changes in approach and reasons for change

“Nothing to Report”

5.B. Actual or anticipated problems or delays and actions or plans to resolve them

“Nothing to Report”

5.C. Changes that had a significant impact on expenditures

“Nothing to Report”

5.D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

“Nothing to Report”

6. PRODUCTS

6.A. Publications, conference papers, and presentations

Parash Parjuli, Thien Ly Nguyen, Purba Singh, Lianna Li, Celine Prunier, Santosh Kumar, Sailaja Eragmerdi, Seval Ozkan, Hao Me, Jussara M. Docarmo, John E. Hall, and Azeddine Atfi. Twist1 Drives Fatty Pancreas Formation and Diabetes (under preparation).

Thien Ly Nguyen, Purba Singh, Parash Parjuli, Lianna Li, Celine Prunier, Santosh Kumar, Sailaja Eragmerdi, Seval Ozkan, Hao Me, Jussara M. Docarmo, John E. Hall, and Azeddine Atfi. Twist1-Driven Fatty Pancreas Formation Facilitates Pancreatitis and Pancreatic Ductal Adenocarcinoma Progression (Abstract presented in Orlando, September 2017, American Association for Cancer, Advances in Modeling Cancer in Mice).

6.B. Website(s) or other Internet site(s)

"Nothing to Report"

6.C. Technologies or techniques

"Nothing to Report"

6.D. Inventions, patent applications, and/or licenses

"Nothing to Report"

6.E. Other Products

"Nothing to Report"

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

- Azeddine Atfi: no change
- Hao Mei: no change
- Parash Parajuli: no change

Name:	Thien Ly Nguyen
Project role:	Graduate Student
Researcher identifier:	79514
Nearest person month worked:	12
Contribution to Project:	Thien Ly performed the histology experiments related to fatty pancreas formation
Funding Support:	Her stipend is fully supported by the Department of Biochemistry, University of Mississippi Medical Center

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

R01CA210911 (NIH/NCI)

4/1/2017 - 3/31/2022

Title: Targeting the TGIF/Twist1 network in osteosarcoma

Role: Azeddine Atfi, Principal Investigator

There is no overlap with DOD **PR152164**

PR162051 (DOD)

4/1/2017 - 11/30/2018

Selected for funding (under negotiation)

Title: Investigating the role of TGIF in beta cell function and diabetes.

Role: Azeddine Atfi, Principal Investigator

There is no overlap with DOD **PR152164**

What other organizations were involved as partners?

“Nothing to Report”

8. SPECIAL REPORTING REQUIREMENTS

8.A. Collaborative Awards:

"Nothing to Report"

8.B. QUAD CHARTS:

"Nothing to Report"

9. APPENDICES

“Nothing to Report”